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Epidermal growth factor (EGF) receptor is expressed in many normal tissues, including mammary epithelium. Overexpression or mutation of the EGF receptor cause neoplastic transformation in many cell types. Several studies have implicated the EGF receptor as a good prognostic marker in breast cancer, but its role in the etiology and progression of this malignancy is still under dispute. We propose to utilize a genetic approach to investigate this issue, by developing transgenic mice in which the EGF receptor gene in the mammary gland will be inactivated at the onset of first lactation. We have made the construct for the gene targeting and have 5 individual embryonic stem (ES) cells with potential correct DNA recombination to generate the loxP mouse. We also have made the final construct to generate white acid protein(WAP)-cre transgenic mouse. We hope to generate the transgenic mouse model next year so that we will be able to study the question whether the EGF receptor plays a functional role in the etiology and progression of mammary carcinoma. The answer to this question would indicate the feasibility of using the EGF receptor as a target in breast cancer therapy.

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Introduction

Breast cancer has been the leading cause of death among non-smoking women and thus has been the focus of intensive research. For the last decade or so, many researchers have concentrated on understanding the molecular basis of breast cancer. Since the epithelial cells of the breast are regulated by a variety of hormones and growth factors, it appears that abnormal hormonal milieu might be one of the critical factors in the development of breast cancer. EGF (epidermal growth factor) is a growth-stimulating factors and act as an autocrine and paracrine growth factor. The expression of both EGF and is positively regulated by estrogen and progesterone receptors [1].

EGF binds to EGF receptor and both epithelial and stromal cells of breast carcinoma express EGF receptor. The EGF receptor is a 170 kDa transmembrane glycoprotein, which belong to the tyrosine kinase receptor family. The role of the EGF receptor in breast cancer has been studied in great detail over the last decade, but it still remains under debate [2]. More than 10,000 breast cancer patients have been assayed for EGF receptor [2]. The expression of EGF receptors on normal breast epithelial cells is low and elevated expression of EGF receptors occurs in about 40% of primary breast cancers. There is a clear reverse relationship between EGF receptor expression and estrogen receptor expression, as well as between the expression of EGF receptor and progesterone receptor [3]. High levels of EGF receptor are associated with poor tumor differentiation, high tumor grade, aneuploidy and high rate of cell proliferation [4]. There is still no consensus, however, on the significance of EGF receptor in breast cancer prognosis or its correlation with the relapse-free survival and overall survival [5,6]. In node-positive patients, the EGF receptor appears to be a good prognostic marker, but in the node-negative patients, the EGF receptor does not appear to have a prognostic value [7,8].

EGF receptor, is detected in a large variety of tissues with the exception of hemopoietic cells. Overexpression of either the ligand of the EGF receptor or the receptor leads to the transformed phenotype in many different cell types [9-15].

The development of the transgenic mouse and gene knockout techniques provides an exceptional opportunity to elucidate the role of the EGF receptor signal pathway in mammary gland cancer [16]. Mouse models have already proven to be useful in mammary gland cancer studies. Thus, overexpression of the ligand of EGF receptor in the mammary gland of transgenic mice under the control of a strong MMTV (mouse mammary

tumor virus) promoter or a matollothionein promoter results in a much higher rate of mammary neoplasia [17-19]. Neoplastic transformation of mammary glands in transgenic mice was also observed when c-myc was overexpressed from the MMTV or WAP (whey acidic protein) promoter [20,21].

The role of the EGF receptor in mammary physiology has been indicated by the analysis of a mutant mouse strain, waved-2 [22]. This strain contains a single nucleotide alteration of the EGF receptor gene at position 743, resulting in a valine to glycine substitution in a highly conserved region of the EGF receptor tyrosine kinase domain. The mutant EGF receptor lacks the high affinity ligand binding and the rate of ligand-dependent internalization is slower than with the wild-type EGF receptor. The phenotype of the waved-2 mouse includes a smaller size of the mammary gland with a reduction of milk in the duct and a less pronounced secretary vacuolation within lobules, as compared to the wild-type mouse. However, it is still unclear whether the abnormal mammary gland of waved-2 mice is due to a developmental problem or to the impaired function of the EGF receptor.

Three groups have generated null mutations of the EGF receptor in mouse by homologous recombination in embryonic stem (ES) cells [23-25]. The phenotype of the null mutation in mouse turned out to be different in different genetic backgrounds. However, no female mice live long enough to develop mature mammary gland. Thus, the null mutation of the EGF receptor in mouse does not provide us with a model to study the role of the EGF receptor in mammary cancer. Therefore, we need to generate a mutant mouse lacking EGF receptor specifically in the mammary gland. Recent success of cre-loxP mediated gene knockout technique [26], which allows one to change the gene of interest in a tissue-specific manner, provides us with the tool to generate mice lacking EGF receptor only in the mammary gland. This mutant mouse will be our model to study the role of the EGF receptor in mammary cancer. We will evaluate the effect of the EGF receptor on the incidence of mammary tumors induced by chemical carcinogenesis. Analysis of this mutant mouse should reveal the role of the EGF receptor in the etiology and progression of breast cancer and will indicate the significance of the EGF receptor as a therapeutic target.

Body

1) Construction of the targeting vector for EGF receptor gene. (Task 1 in statement of work)

In order to obtain the genomic DNA of EGF receptor for the gene targeting construct, we screened a genomic library of a mouse strain 129 with EGF receptor exon 1 probe. The probe is a 232 bp fragment isolated from the PCR product using two primers from published exon 1 of EGF receptor gene sequence (GenBank Accession: X59698). This probe was further confirmed by DNA sequencing. We first screened ~2 million plaques and obtained 10 potential positive clones. After another two rounds of screening, we got 2 real positive clones. Those 2 positive clones were mapped by many different enzyme digestions and by Southern blot analysis. The patterns for all the restriction digest and Southern blot analysis are identical, indicating that the two clones were from the same region of EGF receptor gene. The clone was further confirmed to be EGF receptor gene by DNA sequencing. After mapping the clone, we insert one loxP sequence at XcmI site which is about 2.5 kb in front of the 5'-end of exon 1 of EGF receptor gene. This loxP site is also followed by a NcoI restriction site which will be used to determine the correct DNA recombination after gene targeting. We also inserted the loxP-neo-TK-loxP cassette at AvrII site which is about 0.7 kb of the 3'-end of exon 1 of EGF receptor gene. The orientation of the loxP site and loxP-neo-TK-loxP cassette is checked by Southern blot analysis and DNA sequencing. The final construct is confirmed by several restriction digest and Southern blot analysis. See figure 1 for the final construct.

2) Cloning of the promoter region of WAP (white acid protein) gene and construction of cre-WAP. (Task 2 in statement of work)

To construct the cre gene under the control of WAP promoter, we screened the same genomic library for EGF receptor gene as well as for the promoter region of WAP gene. The probe is a ~500 bp fragment isolated from the PCR product using two primers from the published sequence within the promoter region of WAP (GenBank Accession: X79437). The probe was further confirmed by DNA sequencing. We first screened about 0.8 million plaques and there were 6 potential positive clones. Following another two rounds of screening, we obtained 4 positive clones. Those 4 clones were mapped with many different enzyme digestions and Southern blot analysis. All 4 clones show different digestion patterns, indicating that those 4 clones contain different region of WAP promoter region. The first

clone we analyzed contains only about 1 kb of WAP promoter region by restriction mapping and Southern blotting analysis. Since this clone does not contain enough 5'-end sequences, we decided to analyze another clone. This one contains about 7 kb 5'-end promoter region, but we only isolated about 3.5 kb from it since all the published data suggest that ~3.0 kb of WAP promoter region is enough for mammary gland-specific expression. We had some difficulty to subclone large fragment of WAP promoter DNA. It appears that some kind of DNA recombination occurs in the bacterial, since we can not subclone large fragment fo WAP promoter DNA. After testing many different strain of the bacterial, we were able to find the right bacterial strain ("SURE" from Stratagene) for subcloning WAP promoter region. In order to obtain higher and stable expression of cre protein in the nucleus, we made two modification for the final construct of WAP-cre plasmid. One modification is to add a nuclear target sequences in front of the first protein coding region of cre protein. This modification have shown to improve the percentage of recombination in the mouse by Dr. Klaus Rajewsky who send me the cre gene. Another modification is to add a splicing sequence which was before the coding sequences. This modification also appears to enhance the percentage of recombination. The plasmid for the construct is pCMVβ from Clontech. We replaced CMV promoter region with the 3.5 kb WAP promoter region we cloned, and replaced the β–gal with cre gene containing the nuclear target sequences we got from Dr. Klaus Rajewsky. The original plasmid pCMVβ contains a SV40 splice region. The final construct is confirmed by several restriction digest and southern blot analysis. See figure 2 for the final construct.

3) Electroporation of ES cells with EGF receptor construct. (part of Task 3 in statement of work)

We have introduced the EGF receptor targeting vector we constructed into ES cells by electroporation. After ten days of G418 selection, we have isolated about 500 colonies. Each individual clone was expanded and the DNA from each clone is isolated for Southern blot analysis. The probe we used for Southern blot analysis is a ~500 bp fragment 5'-end outside targeting construct. After analyzing 400 individual clone, we have identify 5 potential positive ones. We are now expanding the ES cells from those 5 positive clones for more DNA to confirmed those ES cells had gone through the correct DNA recombination.

Conclusions:

We have finished the most of the work task that we should for the first year. Although we new do not have any data related to breast cancer, we are moving along with our project very well . Last year we had to waste a lot time for the paper work to obtain the license from DuPont to use the loxP site and cre gene. This year we spend most of the time screening libraries, cloning, subcloning and mapping. Next year we hope will have the transgenic mouse to start our studies related to breast cancer.

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Appendices

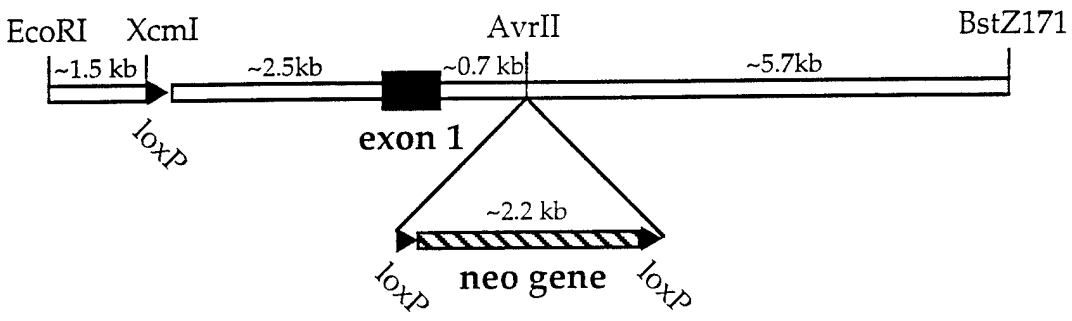

Figure 1. **EGF receptor gene targeting construct**

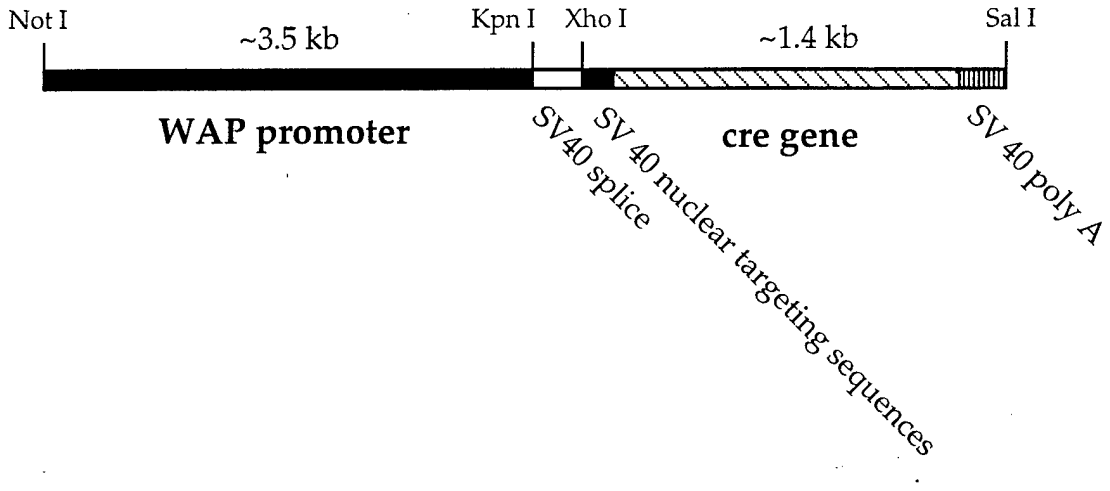

Figure 2. **transgene construct with WAP promoter and cre gene**